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Mycoviruses infecting the forest pathogen *Heterobasidion annosum* - mutual interactions and host reactions

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Academic dissertation

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ABSTRACT

The fungal species complex *Heterobasidion annosum* sensu lato (s.l.) is considered one of the most devastating conifer pathogens in the boreal forest region. They affect European coniferous forests with root and butt rot, causing annual economic losses of €800 million. Despite several efforts in practical forestry to control the disease, the economic loss remains considerable. Therefore, it is still necessary to introduce alternate control measures for *Heterobasidion* infection.

Heterobasidion spp. are infected by a diverse community of mycoviruses, mostly partitiviruses. Here, these viruses were studied to find potential viruses for biocontrol purposes. We described six novel Heterobasidion partitivirus (HetPV) species phylogenetically related to Helicobasidium mompa partitivirus V70 that infect four pathogenic *Heterobasidion* species. Interestingly, our study revealed that HetPV13-an1 causes severe phenotypic debilitation in its native and exotic fungal host. The RNA sequencing of isogenic virus infected and cured fungal strains showed that HetPV13-an1 affected the transcription of 683 genes. The RT-qPCR analysis showed that the response toward HetPV13-an1 infection varied between *H. annosum* and *H. parviporum*. Moreover, the wood colonization efficacy of *H. parviporum* infected by HetPV13-an1 was restricted in living Norway spruce trees.

The ratio of polymerase and coat protein genome segments/transcripts of eight partitiviruses analysed was highly variable in mycelia. All the virus species had unique ratios of the genome segments, which were stable over different temperatures and hosts.

The co-infection with HetPV13-an1 and HetPV15-pa1 reduced host growth up to 95%. Regarding the transmission efficacy of mycoviruses, HetPV15-pa1 transmission to a pre-infected host was elevated from zero to 50% by the presence of HetPV13-an1, and a double infection of these viruses in the donor resulted in an overall transmission rate of 90%. Altogether, the study demonstrated that the interplay between co-infecting viruses and their host is highly complex and that partitiviruses show potential for biocontrol.

Keywords: *Heterobasidion spp.*, wood decay, mycovirus, partitivirus, phylogenetics, RNA transcripts, transmission, growth rate.

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"**Knowledge without practice is like a tree without fruit!**"- (Prophet Muhammad PBUH)

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III. Jurvansuu, J., **Kashif, M**., Vaario, L., Vainio, E. & Hantula, J. (**2014**). Partitiviruses of a fungal forest pathogen have species-specific quantities of genome segments and transcripts. *Virology.* 462-463, 23-33. DOI: 10.1016/j.virol.2014.05.021

IV. **Kashif, M**., Jurvansuu, J., Vainio, J. E. & Hantula, J. Alphapartitiviruses of Heterobasidion wood decay fungi affect each other's transmission and host growth. Accepted (**2019**). DOI: 10.3389/fcimb.2019.00064

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Account on authors' contributions:

I. **KM** wrote most of the manuscript, participated in planning and practical part of the manuscript together with other authors.

II. **KM** partially analysed RNA-seq data, designed some RT-qPCR primers followed by validation of selected DEGs genes from RNA-seq data.

III. **KM** participated in practical part of the research including evaluation of reference gene and growth rate experiment.

IV. **KM** with other authors designed and performed the experiments then analysed the data and wrote the paper.

Other recent articles not part of this thesis:

1. **Kashif, M**., Pietilä, S., Artola, K., Jones, R., Tugume, A. K., Mäkinen, V. & Valkonen, J. P. T. (**2012**). Detection of viruses in sweetpotato from Honduras and Guatemala augmented by deep-sequencing of small-RNAs. *Plant. Dis.* 96, 1430-1437. DOI: 10.1094/PDIS-03-12-0268-RE
2. Ruzicka, K., Zhang, M., Campilho, A., Bodi, Z., **Kashif, M**., Saleh, M., Eeckhout, D., El-Showk, S., Li, H., Zhong, S., De Jaeger, G., Mongan, N., Hejátko, J., Helariutta, Y. & Fray, R. (**2017**). Identification of factors required for m6A mRNA methylation in Arabidopsis reveals a role for the conserved E3 Ubiquitin ligase HAKAI. *New. Phytol.* 215, 157–172. DOI: 10.1111/nph.14586

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ABBREVIATIONS

aa	Amino acid
AbV1	A. bisporus virus 1
BcMV1	*Botrytis cinerea* mitovirus 1
BLAST	Basic local alignment search tool
Bp	Base pair
BpRV1	*Botrytis porri* RNA virus 1
CHV1	Cryphonectria hypovirus 1
CP	Coat protein/ capsid protein
DaRV	*D. ambigua RNA virus*
DEGs	Differentially expressed genes
ds-RNA	Double stranded Ribonucleic acid
FC	Fold change
FgV1-DK21	*Fusarium graminearum* virus 1, DK21 strain
FvBV	*F. velutipes* browing virus
GaRV-MS1	*Gremmeniella abietina* RNA virus-MS1
GC content	Guanin-cytosine content
gDNA	Genomic deoxyribonucleic acid
HetPV	Heterobasidion partitivirus
HetPV13	Heterobasidion partitivirus 13
HetRV6	Heterobasidion RNA virus 6
HvV190S	Helminthosporium victoriae virus 190S
ICTV	International Committee on Taxonomy of Viruses
JGI	Joint Genome Institute
kDA	Kilodalton
LIV	LaFrance isometric virus

MBV	Mushroom bacilliform virus
MyRV1	*Mycoreovirus* 1
nsRNA	Negative-sense RNA
nt/nts	nucleotide(s)
OMIV-1	Oyster mushroom isometric virus 1
Poly(A) tail	Stretch of polyadenosine tail (Polyadenylation)
RdRp	RNA-dependent RNA polymerase
RNAi	RNA interference
RNA-seq	RNA sequencing
RnMBV1	*Rosellinia necatrix* megabirnavirus 1
RnPV1	*Rosellinia necatrix* partitivirus 1
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Reverse transcription-quantitative PCR
s.l.	Sensu lato
s.s.	Sensu stricto
ssDNA	Single-stranded DNA
SsHADV1 virus 1	*Sclerotinia sclerotiorum* hypovirulence-associated DNA
SsHV2	*Sclerotinia sclerotiorum* Hypovirus 2
SsMBV1	*Sclerotinia sclerotiorum* megabirnavirus 1
SsNSRV-1	*Sclerotinia sclerotiorum* negative-stranded RNA virus 1
SsPV1	*Sclerotinia sclerotiorum* partitivirus 1
ss(+)RNA	Single-stranded positive-sense RNA
TMV	Tobacco mosaic virus
UTR	Untranslated regions
YnV1	Yado-nushi virus 1 (yado-nushi = room owner/landlord)
YkV1	Yado-kari virus 1 (yado-kari = borrowing a room to stay)

1. INTRODUCTION

1.1 Boreal forest vegetation and influence of fungal diseases

Of the world's land area covering 13 billion hectares, 4 billion ha is home to natural flora which has been characterized as forest. Forests require sufficient temperature, rainfall, and appropriate location to facilitate best management practices for growth and regeneration of natural vegetation (Rantala, 2011). Conifers play a vital role economically and ecologically in current human civilization. Finnish forests are considered part of the northern area of the boreal coniferous forest zone (around one billion ha), which covers about one quarter of the world's total forested land areas. Common tree species found in Eurasia and North America are members of coniferous genera including pines (*Pinus*), spruces (*Picea*), larches (*Larix*), firs (*Abies*) and broadleaf trees including birches (*Betula*), alders (*Alnus*), willows (*Salix*), beeches (*Fagus*), oaks (*Quercus*), and aspens (*Populus*) (Fagerstedt et al., 2005; Rantala 2011). Finland's forests are considered as the densest in the world, with as many as 90652 trees per km^2 (Crowther et al., 2015; https://stat.luke.fi/en/forest-resources). Finland has a forested area of 26 million ha or 86% of its total land area (Willoughby et al., 2009). The volume of Finnish forest trees includes 50.4 % of Scots pine (*Pinus sylvestris*), 30.1 % of Norway spruce (*Picea abies*), 16.2 % of birch (*Betula pendula* and *B. pubescens*), and 3.5% of other broadleaves (Sevola, 2007; https://stat.luke.fi/en/forest-resources).

Forest ecology in nature shows the complexity of forest structure and dynamics. The life cycle of conifer trees is challenged by several biotic stresses in the form of a disease caused by forest pathogens. A forest disease is a result of biological disorders in the forest that cause modifications in structure and distribution of its vegetation. Overall, fungal diseases infecting forest tree vegetation have been classified based on infections in different parts of the host tree and the nature of the disease. Different fungal diseases include root and butt rots, stem rots, vascular diseases, canker diseases, branch and tip blights on needle tips and cones, and foliar diseases (Gonthier & Nicolotti 2013). In particular, the basidiomycetous fungus *Heterobasidion annosum* s.l. cause huge economic losses in spruces and pines across the northern hemisphere which ultimately leads to losses in tree growth and wood quality (Garbelotto & Gonthier, 2013), with damage exceeding 50 and 800 million euros annually in Finland and Europe, respectively (Woodward et al. 1998; Asiegbu et al., 2005; Finnish Ministry of Agriculture and Forestry 2008). Additive infection is caused by another fungal genus, *Armillaria*, which negatively affects wood quality, and both of these pathogens can cause huge economic losses by reducing timber volumes as a result of growth reduction and mortality (Bendz-Hellgren & Stenlid, 1997; Mallett & Volney, 1999; Turbe et al., 2011; Gonthier & Nicolotti 2013). Moreover, there are other wood rotting fungi such as *Ganoderma* spp., *Hericium* spp., *Laetiporus* spp., *Perenniporia fraxinea*, *Pleurotus* spp, *Schizophyllum* spp., *Stereum* spp., and *Trametes* spp. (Guglielmo et al., 2007).

1.2 Fungal pathogen genus Heterobasidion: taxonomy, biogeography and impacts on practical forestry

Basidiomycete (Bondarzewiaceae) fungal pathogen *Heterobasidion annosum* s.l. species complex is considered as one of the most destructive forest pathogens that causes infectious disease known as root and butt rot in conifers, preferably on spruce and pine trees. Mainly the fungus includes a two species complex known as *Heterobasidion annosum* (Fr.) Bref.

s.l. and *Heterobasidion insulare* (Murril) Ryvarden s.l. The *H. annosum* s. lat. cluster constitutes three European species including *H. parviporum, H. annosum* and *H. abietinum* (Niemelä and Korhonen 1998) and two North American species, i.e., *H. irregulare* and *H. occidentale* (Ostrosina & Garbelotto, 2010) infecting different but overlapping ranges of host tree species. The *Heterobasidion insulare* complex includes mainly saprophytic Asian species (Dai et al., 2003). Though *H. annosum* s.s. mainly causes infection in pines, it can also infect spruce. This shows that both *H. annosum* and *H. parviporum* are able to infect Norway spruce, however, *H. parviporum* colonizes Norway spruce forests as much as 10 times more often than *H. annosum* in southern and western Finland (Korhonen & Piri 1994; Korhonen et al. 1998). *H. irregulare* from the species complex is first fungal strain described for the complete annotated genome sequence and transcriptome which further revealed its dual lifestyles both necrotic by living on its host and saprotrophic by colonizing dead wood (Olson et al., 2012; Garbelotto & Gonthier 2013). Recently, comparative genomics analysis of a reference genome sequence for Norway spruce pathogen (*H. parviporum*) revealed overall genomic variation in the fungal species of Finnish origin (Zhen et al., 2018).

The research work on root rot (*Heterobasidion* spp.*)* was initiated by two German scientists known as Theodor and Robert Hartig. Theodor Hartig first identified the infection of fungi in the trees followed by further aetiology of the disease in accordance with Koch's postulates by his son Robert Hartig (Hartig, 1975; Woodward et al., 1998). Studies show that primary mycelium arise from the germination of a basidiospore producing a haploid homokaryon, whereas secondary heterokaryotic mycelium with two different nuclear haplotypes appear as a result of interaction of two compatible primary mycelia (Korhonen, 1978; Hansen et al., 1993). Moreover, Korhonen (1978) also showed that *H. annosum* s.l. was not a uniform species but a complex of intersterility groups later described as separate species.

Over the years, fungal infection causes no obvious external symptoms except resinous lesions which may appear at the stem or at the base of the tree and the fungal pathogen is able to develop within the stem of the living tree. In the later stages disease development in old spruce trees may cause severe root and butt rot, a less dense deteriorated crown, or a more visible symptom like swollen butt. Moreover, young conifer seedlings (spruce and pine) infected by a fungal pathogen may even die over a season with visible symptoms of red or brown foliage and loss of needles (Woodward et al. 1998; Asiegbu et al., 2005; Garbelotto & Gonthier, 2013). Similarly, infection develops slowly in old pine trees with prior symptoms of significant decrease in annual shoot growth and shading of old needles which ultimately results in a thinner crown. Fruiting bodies of fungal pathogens are generally found at the base of stumps or dead trees (Woodward et al. 1998; Gonthier & Nicolotti 2013). Like other basidiomycetes, the major components of chemical composition of *H. annosum* include carbohydrates, organic acids, fatty acids, amino acids, proteins, nucleic acids, enzymes, and toxins. *H. annosum* spp. can cause wood decay by degrading lignin and cellulose components (Woodward et al. 1998).

The dispersal of fungal spores is reduced by silviculture practices, stump treatment with a biocontrol agent (*Phlebiopsis gigantea*) or urea, and winter cutting, whereas the spread of the pathogen through roots to other healthy trees is restricted by stump removal and more effectively controlled by clear cutting followed by growing resistant trees in tree species rotation or even growing mixed stands of conifer and broadleaves (Piri et al., 1990;

Woodward et al., 1998; Piri, 2003; Lygis et al., 2004; Asiegbu et al., 2005; Garbelotto & Gonthier, 2013).

1.3 Mycovirus and fungal infections

1.3.1 Mycoviruses, their taxonomy and their interaction with the fungal host

Viruses that cause infection in fungi are called mycoviruses or fungal viruses. The viral infection in fungi (mycovirus) was described for the first time in 1962 due to an infection that caused serious disease in *Agaricus bisporus*, a cultivated edible mushroom, resulting in huge economic losses to the mushroom industry (Ghabrial et al., 2015; Son & Kim 2015). Mycoviruses infect a wide range of fungal taxa including Chytridiomycota, Zygomycota, Ascomycota, and Basidiomycota (Ghabrial & Suzuki, 2009; Pearson et al., 2009; Ghabrial et al., 2015). Unlike other viruses, fungal RNA viruses do not possess extracellular infective particles and intracellular transmission occurs through intramycelial contact known as anastomosis and sexual or asexual spores (Ghabrial & Suzuki, 2008; Son et al., 2015; Vainio & Hantula, 2016).

Similar to animal or plant viruses, fungal viruses also require the living host cells to replicate. These viruses are located in the cytoplasm or mitochondria of the host and cause latent or no obvious symptoms; however both adverse and mutualistic effects have been reported (Huang & Ghabrial, 1996; Lakshman et al., 1998; Preisig et al., 2000; Ahn and Lee, 2001; Márquez et al., 2007; Yu et al., 2010; Hyder et al., 2013; Xiao et al., 2014). The most common group of fungal viruses composed of linear dsRNA genomes has been classified into seven families including *Chrysoviridae*, *Endornaviridae*, *Megabirnaviridae*, *Quadriviridae*, *Partitiviridae*, *Reoviridae*, and *Totiviridae* (Ghabrial et al., 2015). However, recent studies based on metagenomics approaches revealed broad range of various types of mycoviruses beyond dsRNA viruses including ss(+)RNA and nsRNA virus genomes, and even rarely found ssDNA virus (Wet et al., 2011; Marzano et al., 2016; Mu et al., 2018; Vainio & Hantula, 2018).

1.3.2 Double-stranded RNA (dsRNA) viruses infecting H. annosum

Fungal viruses occurring as a single or coinfection of more than one viral strain are hosted by about 15-17% of *Heterobasidion* strains (Ihrmark 2001; Vainio et al., 2015b; Vainio & Hantula, 2016). It has been found that a taxonomically unassigned viral species known as Heterobasidion RNA virus 6 (HetRV6) occurs as 70% of all dsRNA vrius infections in European isolates of *Heterobasidion*. Fungal mycelia are also reported to host other viral infections from families including *Partitiviridae* and *Narnaviridae* (Ihrmark, 2001; Vainio et al., 2011a Vainio et al., 2012; Vainio et al., 2015a). It was further described that fungal basidiospores and conidia were infected with dsRNA elements (viruses) (Ihrmark et al., 2002, 2004).

The majority of the viruses isolated from the fungal host *Heterobasidion* spp. belong to genera *Alphapartitivirus* and *Betapartitivirus* of the family *Partitiviridae* for which 18 virus species have been reported (Hantula & Vainio 2016; Vainio et al., 2018). The partitivirus has a dsRNA bipartite genome composed of two independent segments encoding a putative RNA-dependent RNA polymerase (RdRp) and a capsid protein (CP) (Ihrmark, 2001; Nibert et al., 2014; Vainio et al., 2014; Vainio & Hantula, 2016).

1.3.3 Transmission of mycoviruses infecting Heterobasidion spp.

Horizontal transmission of these viruses (Fig. 1) occurs both within and between species of *Heterobasidion* on artificial growth medium (Ihrmark et al., 2002; Vainio et al., 2010; Vainio et al., 2011; Hyder et al., 2013; Vainio et al., 2013). Interestingly, *Heterobasidion* viruses can efficiently cross the borders of vegetative incompatibility of *Heterobasidion* species (homokaryotic or heterokaryotic mycelia) (Ihrmark et al., 2002; Vainio et al., 2010) shown with *C. parasitica* between different VCGs (Rogers et al., 1986; Coenen et al., 1997; Choi et al., 2012). Vainio et al. (2010) showed that virus transmission between *H. ecrustosum* and *H. abietinum* which belong to two different intersterile species complexes (*H. insulare* s.l. and *H. annosum* s.l., respectively) occurs despite cell death in anastomosis. Similarly in the natural environment, the dispersal of viruses (partitiviruses and HetRV6) has been evidently found frequently both within and between species of *Heterobasidion* (Vainio et al., 2011; Vainio et al., 2012).

Vertical transmission of *Heterobasidion* viruses occurs via basidiospores and conidia (Ihrmark et al., 2002, 2004). *Heterobasidion* basidiospores are able to disseminate long distance up to hundreds of kilometers for favorable conditions (Stenlid & Redfern, 1998). Spore-mediated dispersal of *Heterobasidion* viruses has been studied (Ihrmark, 2004). The local spread of HetRV6 virus strain in nature showed that it was the only virus strain detected based on the presence of dsRNA from *Heterobasidion* spores captured from the air (Vainio et al., 2015b). However, it is still unclear whether partitiviruses may also take spore dispersal for vertical transmission (Vainio & Hantula, 2016).

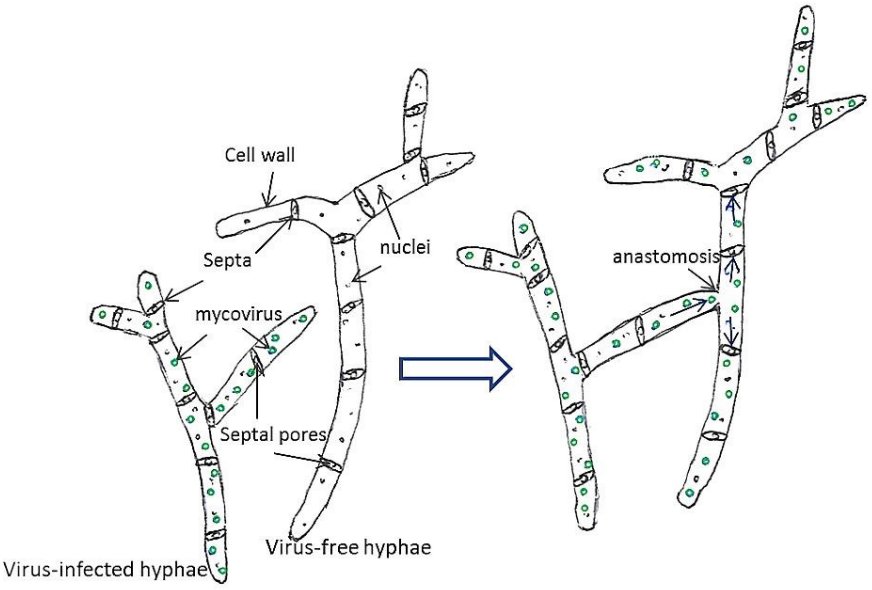

Figure 1. Schematic drawing to show the horizontal transmission of mycoviruses in Basidiomycetous hyphae. After anastomosis, the blue arrows inside hyphae show the movement of virus particles (green circles) in cytoplasm via septal pores. The drawing was made based on ViralZone image (SIB; https://viralzone.expasy.org/1016).

Remarkably, viruses appear to amass in aging centers of *Heterobasidion* clones both via anastomosis and spore dispersal by air (Vainio et al., 2015b). Short-distance dispersal of fungal viruses may also occur through other means such as secondary vectors including mites, beetles or nematodes followed by virus spread to their fungal host (Griffin et al., 2009; Simoni et al., 2014), as shown for *Heterobasidion* viruses (HetPV2 and HetPV6) transmission via pine weevil (*Hylobius abietis*) through viral consistent infection in their fungal host, *H. parviporum* (Drenkhan et al., 2013).

1.3.4 Do the fungal viruses affect their host?

The advent of extensive study related to mycovirus infection on the filamentous fungus *C. parasitica* provided a strong basis for further research in fungal hypovirulence or debilitation in other fungal species (Ghabrial & Suzuki, 2009; Eusebio-Cope et al., 2015). The interaction of cryphonectria hypovirus 1 (CHV1) with its fungal host *C. parasitica* is well studied for hypovirulence and virus/virus interactions (Dawe and Nuss, 2013; Eusebio-Cope et al., 2015). In Europe, Cryphonectria hypovirus 1 (CHV1) infecting isolates of *Cryphonectria parasitica* have successfully been used commercially to control chestnut blight (Nuss, 2005). The infection caused by partitiviruses in different fungal species have been reported to cause variable effects on their growth or hypovirulence (Magae and Sunagawa, 2010; Bhatti et al., 2011; Xiao et al., 2014; Zheng et al., 2014; Zhong et al., 2014, Sasaki et al., 2016). Reduced fungal virulence or hypovirulence is generally connected to phenotypic changes, reduced mycelial growth, and sporulation as a result of virus infection (Hillman et al., 2018).

1.3.4.1 Do viruses infecting *Heterobasidion* spp. affect the fungal host?

Generally, partitiviruses infecting *Heterobasidion* spp. do not cause any visible change or only slightly affect the host, although both adverse and mutualistic effects have been demonstrated (Vainio et al., 2012; Hyder et al., 2013). In addition, unassigned HetRV6-ab6 virus strain caused variable effects when infecting *H. parviporum* or *H. annosum*. The transmitted HetRV6-ab6 caused the decrease or increase in host growth based on temperature conditions and the host (Vainio et al., 2011). More information regarding mycoviruses conferring hypovirulence or debilitation effects are mentioned below in the Table 1.

Table 1. Mycovirus strains appear to cause reduced fungal growth or hypovirulence in their fungal hosts.

Hypovirulent/debilitating mycovirus strains	**Fungal host**	**Hypovirulence symptoms**	**References**
CHV1, CHV2, CHV3, MyRV1 and MyRV2	*Cryphonectria parasitica*	Reduced growth and abnormal pigmentation	Craven et al., 1993; Hillman & Suzuki, 2004; Nuss 2005; Dawe & Nuss, 2013
RnMBV1 and MyRV3	*Rosellinia necatrix*	Reduced growth of fungal colony or slow mycelial growth	Chiba et al., 2009; Kanematsu et al., 2010; Xie & Jiang, 2014

RnPV1 and M dsRNAs	*Rosellinia necatrix*, W8 strain	Reduced mycelial growth and virulence	Sasaki et al., 2006
SsNSRV-1, SsHADV1, SsMBV1, SX466, SsHV2 and SsPV1	*Sclerotinia sclerotiorum, Botrytis cinerea*	Reduced mycelial growth and sporulation, and abnormal colony morphology	Xie & Jiang, 2014; Yu et al., 2010, 2013: Marzano et al., 2015; Xiao et al., 2014; Wang et al., 2015
S. rolfsii BLH-1	*Sclerotium rolfsii*	Hypovirulence and altered phenotypic traits	Zhong et al., 2016
FgV1 and FgV-DK21	*Fusarium graminearum*	Reduced and slow growth, irregular morphology	Chu et al., 2002
Mitovirus 3a-Ld	*Ophiostoma novo-ulmi*	Reduced growth	Deng et al., 2003
HvV190S	*Helminthosporium victoriae*	Reduced fungal growth, hypovirulent phenotype and strong anti-fungal activity	Xie et al., 2016; Huang & Ghabrial, 1996
M2 dsRNA Rhs 1A1	*Rhizoctonia solani*	Reduced virulence, reduced levels of phenylalanine	Lakshman et al., 1998
DaRV	*Diaporthe ambigua*	Hypovirulence-associated traits	Preisig et al., 2000; Smit et al., 1996
BpRV1	*Botrytis porri* (GarlicBc-38)	Reduced mycelial growth and hypovirulence	Wu et al., 2012
BcMV1 and BCMV1-S	*Botrytis cinerea*	Debilitation in phenotypes	Wu et al., 2010
A. bisporus virus 4	*Agaricus bisporus*	severe disease and crop loss	Barton & Holdings 1979
OMIV-1 and OMIV-2	*Pleurotus ostreatus*	Dieback disease	Yu et al., 2003
FvBV	*Flammulina velutipes*	Brown discoloration	Magae & Sunagawa 2010
LFIV, AbV1	*Agaricus bisporus*	La France Disease diseased fruiting body and mycelium	Van der Lende et al., 1994
HetRV3-ec1; partitivirus	*H. ecrustosum* *H. parviporum* *H. abietinum*	Reduced fungal growth, reduced competitive ability	Hyder et al., 2013
HetRV6-ab6	*H. abietinum* host *H. parviporum* *H. annosum*		

Note that all acronyms are defined in abbreviation list.

1.3.4.2 Evolution of mycoviruses

The origin and evolution of mycoviruses have been associated with two proposed main hypotheses. One of which is about ancient coevolution which suggests that despite the unknown origin of mycoviruses, the relationship between fungal viruses and fungal hosts is primitive which leads to the idea of their long-standing coevolution. The other hypothesis relates to plant viruses, suggesting the recent evolution of mycoviruses from plant viruses which further explains that the fungal virus originated from a plant virus which transmitted from plant to fungus within the same host plant (Pearson et al., 2009; Vainio & Hantula, 2016). Previously, Ghabrial (1998) suggested that partitiviruses would have emerged from a totivirus ancestor due to the ancient existence of totiviruses even before the division of fungi and protozoa. Interestingly, alphapartitiviruses comprise closely related clades of viruses infecting fungi and plants, suggesting horizontal transmission across these host groups (Li et al., 2009; Roossinck, 2010, 2018). Consequently, this lateral transmission of mycoviruses may lead to occurrence of viral coinfections of even phylogenetically distant origin (Ghabrial et al., 2002; Tuomivirta and Hantula, 2005; Vainio et al., 2011, 2012; Vainio & Hantula, 2018). This may result in mechanisms of recombination which may influence the evolution of mycoviruses (Liu et al., 2012; Botella et al., 2015).

Viruses are able to adapt and may replicate within the host cells of different species belonging to various kingdoms. It has been shown that brome mosaic virus (BMV) can replicate in the yeast *Saccharomyces cerevisiae* (Panavas & Nagy, 2003). Moreover, a study evidently reports the replication of a plant virus known as TMV in three species of fungal host genus *Colletotrichum* (Mascia & Gallitelli, 2016). Recently, Nerva et al. (2017) showed that mycoviruses (*Partitiviridae* and *Totiviridae*) isolated from a marine fungus harboring the marine plant *Posidonia oceanica* can replicate in plant cells, supporting the evolutionary perspective of mycoviruses switching to plant viruses.

1.3.4.3 Do mycoviruses change the gene expression of the host?

Advanced investigative methods such as RNA sequencing (RNA-seq) have revolutionized the study of infected hosts by facilitating the characterization of RNA transcripts of host or pathogen (Ozsolak & Milos, 2011). RNA-seq analysis of differentially expressed genes (DEGs) in fungal hosts reveals any modifications done by viral infection. Moreover, RNA-seq analysis of genome-based expression of *F. graminearum* transcriptomes revealed varied effects of four taxonomically different mycoviruses, where two viruses seriously altered host genes. (Lee et al., 2014). Vainio et al. (2015) showed that *Heterobasidion* fungi appeared to defend against mycoviruses by using the mechanism of RNA silencing (RNAi). Moreover, a gene annotation study of *H. irregulare* showed that an RNAi mechanism composed of RNase III endoribonucleases called Dicers and catalytic Argonaute proteins occurs in this fungal pathogen (Olson et al., 2012) and in several species of Basidiomycota (Hu et al., 2013).

Mycovirus species of GaRV-MS1 showed recombination via purifying selection (Botella et al., 2015). It has been shown that Cryphonectria parasitica hypovirus 1 (CHV1) has the ability to cause alterations in its host gene expression in several ways. CHV1 virus was found to affect the signal transduction pathway of its host by triggering the expression of RNAi genes including dicer gene *dcl2* and argonaute gene *agl2*. Moreover, this virus also expresses a RNA silencing suppressor gene encoding a papain-like protease *p29* (Chen

et al., 1996; Segers et al., 2007). Kazmierczak et al. (1995) showed that the hypovirus CHV1 can modify the gene expression of the host genes responsible for fungal sex pheromone (Vir1 and Vir2), extracellular laccase (Lac1) and cell wall hydrophobin (Crp).

1.3.4.4 Are fungal viruses interacting with each other?

Viral coinfections of fungi facilitate a system to study different types of virus/virus interactions including synergism related to plants, distinctive antagonistic interactions, and mutualistic interactions among unrelated RNA viruses. Moreover, genome reorganization driven by coinfections can be caused by even simple positive-strand RNA viruses like mitoviruses (Hillman et al., 2018). Synergistic interactions were shown by double infection of CHV1 and MyRV1, which resulted in the increase of MyRV1 accumulation while CHV1 remain unaffected (Sun et al., 2006). In another example, coinfection of viruses (RnMBV2 and RnPV1) was hosted by *Rosellinia necatrix*, where accumulation of RNPV1 increased by approximately two fold (Sasaki et al., 2016; Hillman et al., 2018).

For mutualistic interactions, *R. necatrix* infected by the dsRNA virus named YnV1 which also hosts a capsidless ssRNA virus known as YkV1. The interaction showed that YnV1 is able to replicate independently, whereas YkV1 turned CP of YnV1 away from its replication point and ultimately like dsRNA virus, RdRp of YkV1 replicates to form its own virus particle by capturing CP of its helper virus (Zhang et al., 2016). Antagonistic virus/virus interaction is shown by the key example of *C. parasitica* genes including Dicer-like 2 (*dcl2*) and Argonaute-like 2 (*agl2*), which enable fungal pathogens for antiviral defense. Cyphonectria parasitica hypovirus 1 (CHV1) was found to counteract RNA silencing using genes encoding silencing suppressors. The self-cleavage activity of *p29* caused the release of p29 protein and a basic protein p40 (Hill and Suzuki, 2004). A CHV1 mutant without p29 and p40 (CHV1-Δp69) was shown to affect the replication and transmission of another virus known as RnVV1. Moreover, in *C. parasitica,* RnVV1 was weakened or eliminated by coinfection with MyRV1 or CHV1-Δp69 (Chiba and Suzuki, 2015).

The genome rearrangement of MyRV1 into segments (S1-S4, S6 and S10) during coinfection with CHV1 is related to compromised RNA silencing by the p29 RNA silencing suppressor of CHV1 (Sun & Suzuki, 2008; Eusebio-Cope & Suzuki, 2015). More studies in nature and laboratories have shown genome arrangements in partitiviruses (Chiba et al., 2013a, 2016) and megabirnaviruses (Kanematsu et al., 2014) occurred followed by their introduction by transfection. Mitoviruses and related RNA viruses infecting *Botrytis cinerea* were found interacting with each other in a study which showed that BcMV1 is affected by its associated RNA virus (BcMV1-S) without interfering in the debilitating effects of the virus on its fungal host (Wu et al., 2010). This shows that mycoviruses or their particles may cause an effect while interacting with other viruses or even their host.

2. OBJECTIVES AND HYPOTHESES

The major motivation of this project was to deepen our understanding on the relationship between a basidiomycetous fungus and its virus community; as well as to learn more about the interactions between different viruses within a single mycelium. This is important because such information is very limited on fungal viruses, and therefore will contribute to our understanding of viruses in general.

The specific aims of this study were:

1. To identify and characterize unknown viruses infecting *Heterobasidion* spp. and to identify potential biocontrol agents.
2. To study the potential growth debilitation of HetPV13-an1 virus strain and its effects on its host gene expression.
3. To study the amount of partitivirus RNA transcripts within its fungal host and the effects of host strain and growth temperature on the transcription of viral genes.
4. To determine whether virus infection has an effect on host growth in different *Heterobasidion* strains and temperature conditions.
5. To test whether the phylogenetic relationships of these viruses affect the probability of viral transmission.
6. To investigate the effects of viral co-infections on host growth rate and on the ratio of viral RNA transcripts (RdRp and CP) in the mycelium.

The following hypotheses were tested:

(i) Viruses are able to affect the phenotype of their *Heterobasidion* hosts and the effects can be additive.

(ii) Pre-existing infection within a fungal mycelium lowers the probability of secondary infection by another virus and the strength of this effect depends on the similarity of the two viruses.

(iii) A negatively affecting virus strain has the ability to affect its host's gene expression.

(iv) *Heterobasidion* viruses interact with each other and as a result may affect the quantities of their RNA transcripts.

3. MATERIALS AND METHODS

The materials, methods, virus and fungal strains used in this study are summarized in the Tables 2 and 3, and detailed descriptions can be found in the articles and manuscripts included in this thesis. Therefore, main methods are briefly described here in flow chart (Fig. 2).

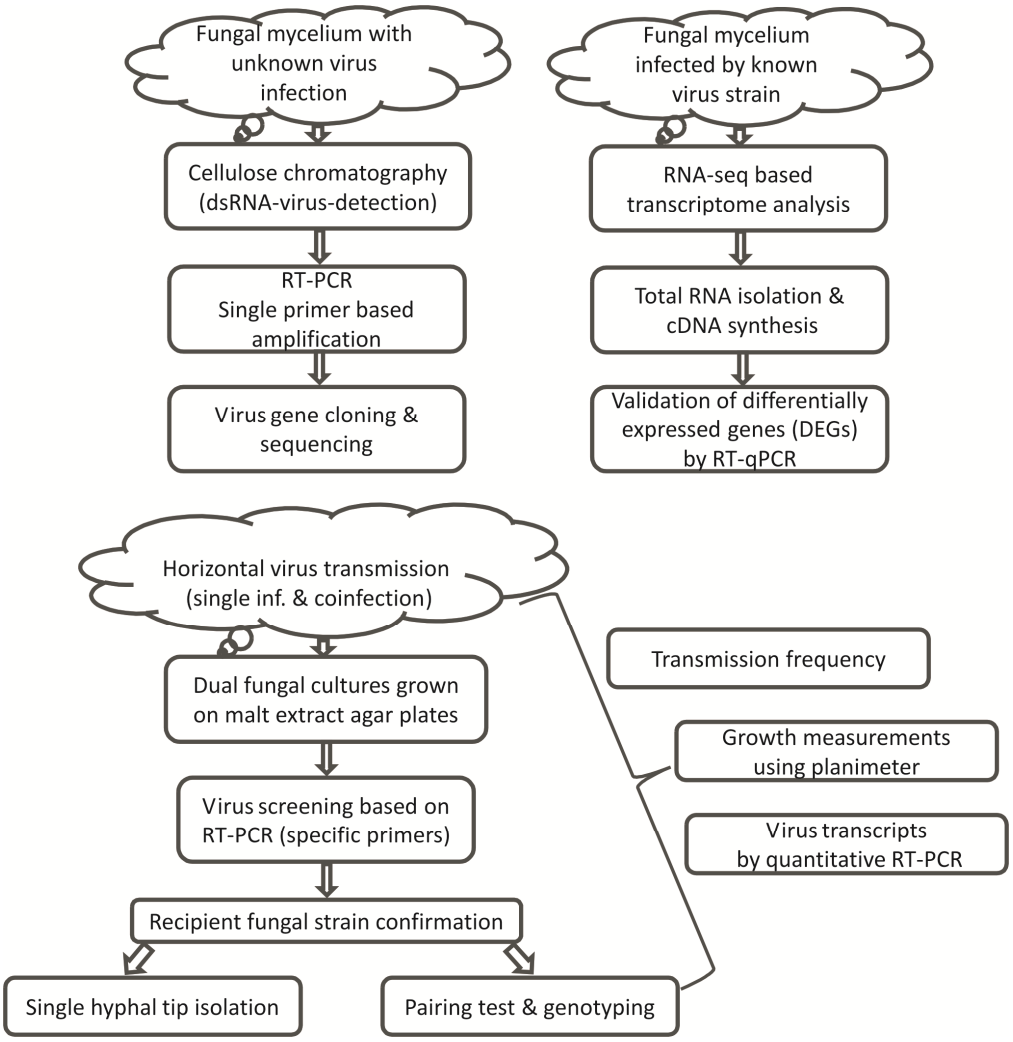

Figure 2. Flowchart for determining different methods used in the study.

Table 2. Summary of the methods used in this study.

Methods	Publications
Cellulose chromatography (dsRNA isolation)	I, II, III
gDNA isolation/PCR	II, III, IV
RNA isolation and cDNA synthesis	I, II, III, IV
RT-PCR	
Sample preparation for RNA-seq and bioinformatics	II
Relative RT-qPCR	
Inoculation of spruce trees in forest plots	
Absolute RT-qPCR (virus transcripts)	III, IV
Fungal growth experiments	II, III, IV
Horizontal virus transmission	

Table 3. Fungal isolates and alphapartitivirus strains and their relevance.

Fungal isolate	**Virus strain/ NCBI accession**	**Origin/ host tree**	**Collector[a] /year**	**Reference**
H. annosum 94221	HetPV12-an1 KF963175-76	Poland *Pinus sylvestris*	PL 1994	This study (I)
H. annosum 94233	HetPV13-an1 KF963177-78			
H. annosum S45-8	HetPV13-an2 KF963179-80	Finland *Pinus sylvestris*	TP, HN 2006	
H. annosum 05003	HetPV13-an3 KF963181-82		HS, KL 2005	
H. parviporum IR-41	HetPV13-pa1 KF963183-84	Finland *Picea abies*	TP 2004	
H. irregulare 57002	HetPV14-ir1 KF963185	USA *Pinus elliottii*	JSB 1957	
H. parviporum 95122	HetPV15-pa1 KF963186-87	Russia *Picea abies*	KK 1995	
H. annosum 03021	dsRNA Virus-free	Finland	KK 2003	This study (IV)
H. annosum 94233/32D	Virus-cured	Poland	none	This study (II)
H. parviporum 06101	HetPV11-pa1 HQ541329, MG948858	Bhutan *Pinus wallichiana*	TK 2006	Vainio et al., 2011a; this study (IV)
H. australe 06111	HetPV11-au1 HQ541328, MG948857			
H. abietinum 93672	HetPV1-ab1 HQ541323-24	Greece *Abies cephalonica*	PT 1993	Vainio et al., 2011a
H. occidentale 98004	Virus-free	USA *Picea engelmannii*	DG 1998	

H. annosum Ha_JH	Virus-free	Finland *Pinus sylvestris*	JH 2006	
H. parviporum 7R18	HetPV2-pa1 HM565953-54	Finland *Picea abies*	TP 2005	Vainio et al., 2011b
H. ecrustosum 05166	HetPV3-ec1 NC_038835-36	China *Pinus massoniana*	KK, YCD 2005	Vainio et al., 2010

a PL P. Lakomy; TP T. Piri; HN H. Nuorteva; HS H. Schneider; KL K. Lipponen; JSB J.S. Boyce Jr.; KK K. Korhonen; TK T. Kirisits; PT P. Tsopelas; DG D. Goheen; YCD Y.C.Dai; JH Jarkko Hantula.

4. RESULTS AND DISCUSSION

4.1 Identification and genomic characterization of novel Heterobasidion partitivirus species (I, IV)

The main objectives of this research were to identify the unknown mycovirus strains from different species of the *Heterobasidion* complex and genomic characterization of novel virus species. Later, potential viruses were observed for effects on the phenotype of their fungal host. Complete partitivirus genome sequences were characterized from isolates 94221, 94233, S45-8 and 95122, 06111, 06101, and partial sequences were determined for isolates 57002, 05003 and IR41. Novel partitivirus species were found that infect three *Heterobasidion* species, which are fungal pathogens of conifers, including *H. annosum, H. parviporum* and *H. irregulare*. We identified four distinct putative partitivirus species: HetPV12, HetPV13, HetPV14 and HetPV15.

The UTR terminal sequence regions of the two genome segments from each virus species were found to be highly conserved (81-90.3%) in the 5´ UTR and less conserved (21.7-27.9%) in the 3´ UTR (I, Table 1). Moreover, previously described virus strain HetPV11 (previously named as HetRV1) for the RdRp genome segment (Vainio et al., 2011) was hosted by *H. australe* strain 06111 of the *H. insulare* complex and HetPV11-pa1 by *H. parviporum* 06101 of the *H. annosum* complex, was characterized for the CP segment in this study. The length of the smaller genome segments were 1818 bp (HetPV11-pa1) or 1819 bp (HetPV11-au1) including 3'-terminal poly(A) tracts. They encoded for a putative CP of 495 aa (nts 124-1611) with a predicted *M*r of 53.4 kDa and GC content of ca. 52%. The larger segment of HetPV11-pa1 and HetPV11-au1 is coding a putative RdRP (2033 and 2029 bp, respectively) (Table 1). HetPV11-au1 is hosted by *H. australe* strain 06111 of the *H. insulare* complex and HetPV11-pa1 by *H. parviporum* 06101 of the *H. annosum* complex (Vainio et al., 2011a).

Other partitviruses have also been reported for their high conservation of terminal regions (Tuomivirta et al., 2003; Lim et al., 2005; Hacker et al., 2006) which may play a vital role in the recognition of RdRp in virus replication (Buck, 1996). All virus species included an interrupted or continuous poly (A) tail at the 3'-end of genome segments (I, Table 1).

According to ICTV, the species demarcation criteria for partitiviruses are ≤ 90% aa-sequence identity in the RdRp and/or ≤ 80% aa-sequence identity in the CP (Nibert et al., 2014). One virus strain was considered for each of HetPV12, HetPV14 and HetPV15, whereas there were four conspecific strains of HetPV13 (HetPV13-an1, HetPV13-an2, HetPV13-an3 and HetPV13-pa1) with high sequence identity (97.2-98.3% RdRp and 94.8-97.5% CP identity at nt level) (I, Table S5). Moreover, regarding CP identity based on complete protein sequences, virus strains HetPV12, HetPV13, HetPV14 and HetPV15 shared 52.6-67.6% identity with RdRp. Notably, HetPV12-an1 and the previously described HetPV3-ec1 had significantly higher (73.7%) CP sequence identities at the protein level, suggesting a close phylogenetic relationship between the two viral species (I, Table S5) which were collected from two different *Heterobasidion* species clusters and belong to different continents (Europe and Asia).

4.1.1 Phylogenetic and dispersal relationships of described partitivirus species (I)

The Bayesian RdRp and CP dendrograms (I, Figs. 1, S2) and the neighbor-joining dendrogram based on RdRp nucleotide sequences (I, Fig. S3) confirmed the close relationship between the conspecific HetPV13 strains and HetPV12-an1 and HetPV3-ec1 were found to be closely related. All of the species characterized in this study (I) associated with the genus *Alphapartitivirus*. These analyses show that there seems to be no geographical or phylogenetic differentiation among viruses related to HetPV3, which agrees with the view that Heterobasidion partitiviruses (HetPVs) are globally widespread (Vainio et al., 2011a).

Based on BlastP, H. mompa partitivirus V70 (HmPV-V70; Osaki et al., 2002) was found to be the only one closely related to described virus species with 57-67% polymerase identity at the protein level (Table S3). Notably, *Heterobasidion* and *Helicobasidium* are significantly different phylogenetically, and *Helicobasidium* is a member of order Helicobasidiales, while *Heterobasidion* belongs to order Russulales. All other available partitivirus sequences, including those from *Heterobasidion* spp., were found to be more distant (less than 43% of polymerase sequence identity). Globally dispersed partitivirus lineages of closely related taxonomical groups of these viruses are widespread and diverse, suggesting that either these lineages are ancient or fungal viruses are more uninhibited in their hosts than commonly thought (Feldman et al., 2012). Another prospect could be including HmPV-V70 within the HetPV3-related virus clade. The transmission of viruses at the interspecies level is found to be a rare occurrence among fungal viruses. Previous studies by Ihmark et al. (2002, 2004) showed that certain HetPVs transmitted from *H. parviporum* to *H. annosum* and *H. occidentale* via hyphal contacts. Moreover, Vainio et al. (2010) found that HetPV3 can be readily transmitted from *Heterobasidion ecrustosum* to *Heterobasidion abietinum* and *Heterobasidion occidentale*. Moreover, nearly identical strains of HetPV11 infecting *H. parviporum* and *H. australe* within the same region of Bhutan, suggest that there is natural inter-species transmission (Vainio et al. 2011a).

According to ICTV, previously published HetPVs can be classified into two main phylogenetic groups: the virus species HetPV1, HetPV3, HetPV4 and other *Heterobasidion* viruses (HetPV5 and HaV) belong to the genus *Alphapartitivirus*, while HetPV2, HetPV7, HetPVP and HetPV8 are grouped in *Betapartitivirus* (Fig. 1; Nibert et al., 2014). In this study, phylogenetic analysis showed a closely linked clade including the newly characterized virus species together with HetPV3 and HmPV-V70. These findings support the idea that one of the HetPV3-related partitiviruses originally found only in the East Asian *H. insulare* strain belongs to a globally distributed virus group occuring at low frequency in *Heterobasidion* species in Europe, East Asia and North America.

The considerable diversity of these viruses enabled us to group the viruses into five putative species, three of which are reported here for the first time with their complete sequence and one with only its RdRp sequence.

4.2 The effects of virus strains on the growth of their fungal host

4.2.1 Severe growth debilitation by Heterobasidion Partitivirus 13 (HetPV13-an1) (II)

This study (II) aimed to investigate the hypovirulence like effects of alphapartitivirus HetPV13-an1 from *H. annosum.* The virus causes serious growth reductions and major modifications in the gene expression of its natural fungal host and other sensitive hosts. Moreover, its effects on the growth and wood colonization efficacy of *H. parviporum* were also studied.

HetPV13-an1 causes exceptionally low growth rate of its natural fungal host and it adversely modifies its host (94233) phenotype as abnormal mycelial morphology in the form of multiple hyphal branching (II, Fig 1.AB). The fungal host was cured (94233/32D) by thermal treatment (32°C) which then showed normal fungal morphology. The particular phenotype of HetPV13-an1 provides the initial evidence for hypovirulence like negative effects on its host.

4.2.2 Heterobasidion partitivirus infection affected by temperature and a new host (III & IV)

In this study (III), the phenotypes of four strains (HetPV1-ab1, HetPV2-pa1, HetPV12-an1 and HetPV3-ec1) of partitiviruses infecting their native and exotic (North American fungal strain) *Heterobasidion* hosts were tested in different temperature conditions. The growth of virus-free and virus infected native fungal strains (5 to 28 days depending on their growth rate) were analyzed at different temperatures. Expectedly, very slow growth was observed for all the strains at 6°C. Three fungal strains, *H. abietinum* (93672), *H. parviporum* (7R18), and *H. ecrostusum* (05166) grew faster at 25°C than at 20°C. Overall, the virus-free and virus containing isogenic pairs grew at almost the same rate except when virus infected 7R18 and 94221 showed slightly reduced growth at 6°C (III, Fig. 3).

Furthermore, growth of the new host *H. occidentale* (98004) with or without virus infection was also studied. As a result, partitivirus strains were found to frequently affect the growth of the new host in all temperature conditions except at 6°C for HetPV2-pa1 and HetPV3-ec1. Comparatively, HetPV1-ab1 decreased the growth of the new host consistently at all temperature conditions. Generally, the new host appeared to have higher vulnerability to the virus infections.

4.2.3 Growth debilitation effects by HetPV13-an1 coinfecting with other partitivirus strains (IV)

The growth rate effects were analyzed for partitiviruses transmitted to recipient host (94233) in 12 parallel independent experiments for each viral infection. The growth rates of fungal host infected by single HetPV13-an1 when compared to the coinfections with HetPV15-pa1 showed that there was consistent, highly reduced growth up to 95% which was found to be the same as with naturally infected HetPV13-an1 in its native host (94233) with growth reduction of 96%. However, growth reductions caused by newly infected HetPV13-an1 ranged from 87-89% (IV, Fig. 2A). Interestingly, these findings of significant debilitating growth effects by coinfection (HetPV13-an1 and HetPV15-pa1) correlate with their significantly enhanced transmissions (IV, Table 3) to the partitivirus-free host

(94233). These findings correlate with previous results showing that HetPV13-an1 caused a serious disease on both *H. annosum* and *H. parviporum*, and has a significant effect on their gene expression (II), and that these two viruses belong to the same phylogenetic clade among alphapartitiviruses (I).

Moreover, HetPV13-an1 coinfected with HetPV11-pa1 showed significant growth reductions up to 88% with a high range of variation in 6 of 12 subcultures due to patchy or slow and fast growing sectors. However, HetPV13-an1 co-infected with HetPV11-au1 had none or very little effect on its host (IV, Fig. 2B).

4.3 HetPV13-an1 causes alterations in the gene expression of the fungal host (II)

The effects of host transcription by HetPV13-an1 infection was analyzed by RNA sequencing (RNA-Seq) of two isogenic strains of *H. annosum* 94233 with and without the viral infection of HetPV13-an1. Illumina sequence reads were aligned to the reference genome, *H. irregulare* V.2.0 (Olson et al., 2012) available at the JGI. The expression of a total of 683 genes was affected by the infection of HetPV13-an1. The 60% (409) of all DEGs were downregulated with as low a FC of 1388, whereas 276 DEGs were upregulated with up to 871 FC.

Generally most of the upregulated DEGs were related to amino acid metabolism (9% of transcripts), citric acid cycle and redox (29%), mitochondrial energy production (5%), and chaperones (II, FIG 3. A), whereas most DEGs of carbohydrate metabolism (20%), RNA related (7%), cell cycle control (8%), cell wall and membrane (10%), sex and compatibility (3%), and DNA damage were found to be significantly downregulated (II, FIG 3. A). RT-qPCR based validation data showed that cell cycle control and sex related DEGs were probably knocked out due to extremely low FCs at -593 and -1388, respectively. Moreover, an analysis of gene expression (RT-qPCR) of the same DEGs for the other fungal host (*H. parviporum*) infected with the virus and without agreed with up to 71% of expression of genes. This showed that the expression of most genes was highly similar, but one third (or 29%) responded differently to the presence of HetPV13-an1 (II, Table 1; Fig 4). It has previously been shown that viruses associated with hypovirulence like *Cyphonectria parasitica* hypovirus 1 (CHV1) are able to alter the gene expression of their host in various ways. The CHV1 virus influences its host signal transduction pathways by inducing the expression of dicer gene *dcl2* and argonaute gene *agl2*. In addition, the virus encodes a papain-like protease p29 which act as a RNA silencing suppressor (Chen et al., 1996; Segers et al., 2007). Contrarily, in our study we did not find any strong response in annotated genes of the RNA silencing pathway, perhaps due to the nature of virus-host interaction and the basidiomycetous host. Four phylogenetically different mycoviruses infecting *Fusarium graminearum* (FgV1-4) generated distinct changes in host transcriptomes, however obvious gene expression changes did not always appear as phenotypic effects (Lee et al., 2014). Similarly, FgV-ch9 infecting *F. graminearum* did not show disease symptoms despite having the virus in large amounts and this effect was further revealed to be due to extremely low expression of the *vr1* gene (Bormann et al., 2018). In this study, HetPV13-an1 caused gene expression related alterations in many basic cellular functions connected with a severely debilitated host phenotype including carbohydrate metabolism, chaperone functions, fungal self-defense and cell cycle control. Interestingly, both HetPV13-an1 and the other partitivirus HetPV3-ec1 tend to produce significantly higher amounts of polymerase transcripts than capsid, suggesting that it may

interfere with cellular processes by additive adverse viral effects (Hyder et al., 2013; Vainio et al., 2015; III). These findings correspond to our other study which shows that HetPV13-an1 consistently produces higher amounts of RNA transcripts and even with selective coinfections was able to cause debilitating effects on the host (IV, Fig 4 and 5). However, HetPV11 strains also produced higher RdRp than CP amounts without causing any negative effects on their host probably due to their nature and phylogenetically distant and different viral species.

4.4 Transmission of selected conspecific and distant alphapartitiviruses

One of the objectives of this study was to determine the effect of interactions on the transmission between two *Heterobasidion* strains using pairs of four viruses with different taxonomic relatedness.

4.4.1 Transmission of HetPV13-an1 across multiple Heterobasidion host strains and growth debilitation by HetPV13-an1 in spruce trees (II)

HetPV13-an1 was successfully transmitted across one homokaryotic and other eight heterokaryotic strains of *H. annosum*, however the virus could not transmit to the other 13 strains of the *Heterobasidion* complex. Different host strains infected by HetPV13-an1 showed variation in growth reductions (II, Fig 2A). Notably, one heterokaryotic and two homokaryotic strains of *H. parviporum* (242-05, RK15A and 109-05) showed significant growth reductions due to viral infection.

The testing of the wood colonization efficacy of *H. parviporum* strain RK15A infected with or without HetPV13-an1 using 46 large living spruce trees was conducted. Following inoculation, after two growing seasons the trees were cut down and wood discs were analysed for the area covered by *Heterobasidion* conidiophores after incubation in plastic bags. The number of trees with *Heterobasidion* infections with and without HetPV13-an1 were 20 and 22, respectively. Interestingly, different tree clones showed variable susceptibility to fungal infection. The growth of *H. parviporum* was analysed above inoculation spots in number of trees were 3 and 8 trees (15% and 36%) (II, Fig. 2B) corresponding to areas of wood colonization that were 36.5 and 133.5 cm^2, respectively ($P = 0.067$) (II, Fig. 2C).

4.4.2 Transmission of alphapartitivirus strains to virus-free and pre-infected isolates (III, IV)

Transmission of four alphapartitiviruses including two conspecific strains of HetPV11 (HetPV11-au1 and HetPV11-pa1; 99% RdRp amino acid similarity) and two relatively closely related viral strains, HetPV13-an1 and HetPV15-pa1 (68% similarity based on RdRp amino acid), was tested in 20 individual experiments for each virus transmission. It was found that HetPV13-an1 had a transmission frequency of 25% to a partitivirus-free host (94233/32D), whereas HetPV11-au1 and HetPV11-pa1 transmitted with 45% and 65% frequencies, respectively (IV, Table 2). HetPV15-pa1 failed to transmit in any of 20 independent experiments. However, transmission frequency of HetPV15-pa1 rose from zero to 50% and 60% when the recipient was pre-infected with HetPV13-an1 and HetPV11-au1, respectively. Moreover, the transmission frequencies of HetPV13-an1, HetPV11-au1 and HetPV11-pa1 to the HetPV15-pa1 infected recipient were 40%, 75% and

50%, respectively (IV, Table 2). This shows that HetPV15-pa1 appeared to require other virus strains as co-helpers to transmit in laboratory conditions. Similarly, it was found that Mushroom bacilliform virus (MBV) may require a helper-virus LaFrance isometric virus (LIV) for its efficient transmission (Romaine and Schlagnhauffer, 1995).

HetPV11-au1 did not transmit to a pre-infected recipient with HetPV11-pa1 and vice versa. The recipient host pre-infected with HetPV11-au1 enhanced transmission of HetPV13-an1 and HetPV15-pa1 and vice versa, otherwise HetPV11-pa1 had no significant effects (IV, Table 2). The results suggest that conspecific HetPV11 strains mutually interfered and hampered one another's transmission between two mycelia of *H. annosum* (94233) when present as a pre-existing infection (Vainio et al., 2015b), however, both viruses exhibited absolutely different effects on transmission when distantly related virus strains are transmitted to a host pre-infected with HetPV11 strains.

Additionally, transmission trials of a double infected host (03021) to a partitivirus-free recipient (94233) were conducted including HetPV15-pa1 coinfected with one of three viruses (HetPV13-an1, HetPV11-au1 and HetPV11-pa1). No transmission was observed for co-infection of HetPV15-pa1 with HetPV11-au1 or HetPV11-pa1 strains, even after 20 repeated experiments. Conversely, transmission of HetPV15-pa1 coinfected with HetPV13-an1 elevated up to 90%, including 75% frequency for transmission of both virus strains (IV, Table 3). In another study (III), three virus strains (HetPV1-ab1, HetPV2-pa1 and HetPV3-ec1) were successfully transmitted to a virus-free new exotic host, *H. occidentale* (98004) which infects tree species of different geographical origin and the new host may not be sharing viral co-evolution. This study shows that horizontal transmission of viruses is not only affected by their interaction with a new host (III) or reintroduction of the virus (HetPV13-an1) to its native host but also affected by pre-existing viruses (IV). A previous study showed that Sclerotinia sclerotiorum mycoreovirus 4 (SsMYRV4) modifies the transcription and phenotype of the host fungus so that somatic incompatibility becomes leaky (Wu et al., 2017). Previous studies have shown that transmissions are common among *Heterobasidion* strains in laboratory and nature (Ihrmark et al 2002; Vainio et al., 2010; Vainio et al., 2013; Vainio et al., 2015; Vainio et al., 2017).

4.5 The amounts of genome and RNA transcripts of partitiviruses infecting *Heterobasidion* spp.

4.5.1 Heterobasidion partitivirus strains have a particular ratio of CP to RdRp in genome segments (dsRNA) (III)

This part of the study was conducted to determine the amounts of partitivirus RNA in host fungi and how they are affected by temperature conditions. The first part of study (III) included relative amounts of genome segments of four partitiviruses across different species of *H. annosum* and *H. insulare* in their natural hosts and in a new host grown in different temperature conditions. Virus hosts were grown at 20°C and 25°C followed by dsRNA isolation based on CF11 affinity chromatography. The dsRNA genome segments were further analyzed by absolute RT-qPCR. Three virus strains (HetPV1-ab1, HetPV2-pa1 and HetPV12-an1) had more CP than RdRp genome segments and the CP to RdRp ratio remained the same in two temperature conditions. Conversely, HetPV3-ec1 from *H. ecrostosum* (05166) had an exceptionally higher amount of RdRp than that of CP, 125 times at 20°C and 12 times at 25°C (III, Fig. 1B). This shows that CP to RdRp ratio is not

always mechanically protected which may cause a more random and biased distribution of genome segments in hypha. This shows that partitiviruses infecting the *Heterobasidion* species complex produce uneven amounts of CP and RdRp genome segments; however their genome segment ratios generally remain persistent in different temperature conditions.

A previous study shows that genome ratios of viral infections in different subcultures were found to be variable (Chiba et al., 2013b). Moreover, Penicillium stoloniferum partitiviruses S produce around twice as many CP encoding particles than particles encoded by the RdRp genome segment, based on CsCl density gradient centrifugation (Buck and Kempson-Jones., 1973). Certain partitivirus (HetPV1-ab1, HetPV2-pa1 and HetPV12-an1) strains infecting *Heterobasidion* spp. mentioned in this study generally produced more abundant CP segments than RdRp which may correspond to a partitiviral particle made of 120 CP and only one RdRp molecule. HetPV genome segment ratios were generally found to be constant but it may also lead to genomic instability for certain viral species as fluctuations shown in the new host. Therefore, variations may suggest that these viruses may require some adjustment period to adapt to a new host fungus.

4.5.2 Heterobasidion partitivirus RNA transcripts affected by temperature and pre-existing virus strains (III & IV)

The amounts of partitivirus RNA in host fungi and how they are affected by temperature, host and pre-existing virus infection were analysed, and whether the amounts of viral RNA influence host growth or vice versa. In the first study (III), the relative amounts of transcripts of four partitiviruses across different species of *H. annosum* and *H. insulare* in their natural hosts and in a new host grown in different temperature conditions were studied. Generally like viral genomes, higher amounts were found of CP than RdRp transcripts in all temperatures 6°C, 20°C, and 25°C (III, Fig. 2A). Interestingly, HetPV3-ec1 showed expression of viral transcripts at an almost equal ratio at 20°C and more of CP than RdRp transcripts at 6°C, however at 25°C the virus produced significantly higher amounts of RdRp transcripts. This showed that at the transcript level each virus strain reacts to different temperature conditions in a distinctive manner (III, Fig. 2B). Moreover, the expression levels of transcripts of three virus strains transmitted, namely HetPV1-ab1, HetPV2-pa1 and HetPV3-ec1, into the new exotic host (*H. occidentale*) showed almost the same correlation as with transcript data from the native host, except that HetPV3-ec1 produced slightly higher amounts of RdRp at 20°C (III, Fig. 4C). This showed that like the native host, temperature conditions do not affect the ratio of viral genome segments in the new host (III, Fig. 4AB). The CP to RdRp transcript ratios of the three virus strains in their natural and new host showed correlations and responded to the temperatures consistently (III, Fig. 4D).

We also studied (IV) the relative amounts of CP and RdRp transcripts of four virus strains (HetPV13-an1, HetPV15-pa1, HetPV11-au1 and HetPV11-pa1) by making comparisons of single infections with coinfections of each virus strain with HetPV13-an1. The amounts of HetPV15-pa1 transcripts remained, on average, around the same in single and coinfection, but two independently created isolates showed variations (IV, Fig. 3A1). Conversely, the amounts of transcripts of HetPV13-an1 were reduced up to 4.8 and 4.6 fold for RdRp and CP in coinfection with HetPV15-pa1, respectively. The CP to RdRp transcript ratio of HetPV15-pa1 remained almost the same in single infection and showed little variation in coinfection. Similarly, there were no significant changes in transcript

ratios for HetPV13 in single and coinfection (IV, Fig. 3A2). Wu et al. (2010) showed that the replication of Botrytis cinerea mitovirus 1 (BcMV1) is suppressed by an associated RNA virus (BcMV1-S), however it did not influence the debilitation effects on *B. cinerea* caused by BcMV1.

Moreover, coinfection of HetPV13-an1 with HetPV11 strains were analyzed for relative amounts and ratios of CP and RdRp. Comparisons of transcript amounts in coinfection to single infection revealed that HetPV11-pa1 expressed reduced levels of transcripts up to 9.6 and 40 times for CP and RdRp, respectively, whereas transcript levels of HetPV13-an1 were reduced only by 2 and 5 times for CP and RdRp, respectively. The relative ratio of CP to RdRp of single infections changed, on average, from 12.5% to 2.5% for HetPV1-pa1 and showed no significant change for HetPV13-an1 (IV, Fig. 3B). Otherwise, viral transcripts in coinfection with HetPV13-an1 and HetPV11-au1 produced reduced amounts of transcripts by 4 times for both RdRp and CP, however HetPV13-an1 produced 1.3 and 2.5 fold reduction in CP and RdRp transcripts, respectively. There was unclear fluctuating change for CP to RdRp relative ratios of both virus strains in single and coinfection due to huge variation in expression of transcripts (IV, Fig. 3C).

Transcript ratios of most viruses had a distinctive response to temperature conditions. The amount of virus transcripts may be described by two pathways including assembly and stability of virus particles in the host cytoplasm. Viral transcript ratios are more temperature dependent than consistent genome segment ratios as the latter depend on temperature, host conditions and virus particle activity.

It is assumed that not only the RdRp segment but the whole particle may be mainly involved in controlling transcription so that the partitivirus may transcribe inside the virus particle. Pan et al. (2009) showed that there is interplay between unstructured domains of partitivirus coat proteins with viral RNA inside the capsid protein.

The formation of partially purified virions contains small amounts of particles known as ssRNA molecules and large amounts of heterogeneous dense particles. These dense particles are involved in the replication cycle which includes the individual genomic dsRNAs with ssRNA tails of variable sizes and particles with one molecule of each dsRNA and ssRNA transcript, and two molecules of dsRNA. However, only particles containing dsRNA are transcriptionally active (Buck, 1978; Ghabrial et al., 2008). Viral genome segments may have connections to the regulation of the amounts of viral transcripts. For instance, the 5´-ends of the two genome segments of the same partitivirus segments share high similarity. In our study (III/IV), the identity in the first 120 nucleotides of CP and RdRp ranges from 64-72%, which is in accordance with observations in other partitiviruses (Strauss et al., 2000). Furthermore, many partitiviruses possess a poly(A) tail in their genomic 3`-end to facilitate the viral proteins to coordinate with viral RNA and communicate with their transcription and packaging into virus particles (Strauss et al., 2000; Lim et al., 2005). Similarly, partitiviruses from this study have on average longer poly(A)-tails for CP than RdRp segments (III, Suppl. Table A1). Therefore, compared to RdRp transcripts, the longer poly(A)-tail may provide more stability to CP transcripts in the host cytoplasm.

5. CONCLUSION AND FUTURE PROSPECTIVES

Closely related strains of HetPV13 were found on *H. annosum* and *H. parviporum* which suggests that virus species might have been transmitted recently across these two fungal species. Moreover, two almost identical HetPV13 strains were identified from *Heterobasidion annosum* from Finland and Poland which shows that the dispersal capacity of these partitiviruses is high. Furthermore, the diversity and ecology of partitiviruses infecting *Heterobasidion* fungi can be investigated by screening fungal collections for viruses at a large scale by employing modern techniques such as next generation sequencing or RNA-sequencing.

The phenotypic debilitation of the fungal host caused by HetPV13-an1 was visible in both homokaryotic and heterokaryotic fungal strains of *H. parviporum* and *H. annosum*. Therefore, the negative effects of the virus strain do not depend on the host species or nuclear condition but on the genetic variation of its host. The growth debilitation effect by HetPV13-an1 was found to be in accordance with the poor wood colonization capability of the fungal host in living trees. RNA-seq *de novo* transcriptomic profiling of *H. annosum* infected by HetPV13-an1 showed that certain pathways of the fungal life cycle were hampered by viral infection. What would be the level of tolerance and stability of the viral effect in response to infection by HetPV13-an1? To address this research question, large number of *H. annosum* s.l. fungal strains need to be investigated to test the infection responses to HetPV13-an1. Moreover, combination of more phylogenetically related partitivirus strains may add value in consistent debilitation effects on the host fungal growth. This may pave the way to develop a biocontrol strategy to restrict the disease spread in infected forest sites.

Heterobasidion partitiviruses possess a distinctive ratio of genome fragments and respond to host growth temperature in a unique manner which shows that partitiviruses depend on their host. This also suggests that these viruses existing without extracellular particles needs to evolve and adapt to survive in the host condition, that's why latent infections by these partitiviruses in their native *Heterobasidion* hosts come to affect new hosts.

The interactions between partitiviruses infecting *Heterobasidion* spp. make a complex relationship with each other. The transmission of different virus strains in different host species of the *Heterobasidion* species complex can be studied further to explore the adaptability of virus strains across host species and their ecological role in fungal infection in nature. In addition, the particular scenario of combined effects of two related partitiviruses (HetPV13-an1 and HetPV15-pa1) shows considerably high transmission efficiency and cause an altered host phenotype. In conclusion, ultimately this provides us an opportunity to develop a biocontrol agent against *Heterobasdion* spp. by testing this coinfection in bioassays and by developing more efficient application strategies in *Heterobasidion* infected forest sites.

Many research questions remain open about the interaction of virus, fungal host, and their interaction with the host tree species. What is the wood decay capability of fungal isolates infected by a virus? What would be the effectiveness of virus transmission from the donor strains into native *Heterobasidion* strains and how to develop practical inoculation methods? Further investigation is needed on the mechanism behind the vertical or

horizontal transmission of *Heterobasidion* viruses which make them cross borders of fungal host species belonging to different genetically defined vegetative compatibility (vc) types.

What would be the implication of testing single virus infection by HetPV13-an1 and coninfection with other homo/heterologous partitiviruses in different biological assays under different conditions? How are the amounts of virus transcripts regulated in its host? The distribution of virus titers or amounts among different zones in the mycelium (patchy or slow and faster growing regions) and at stages of young and old hyphae can also be studied. Moreover, there is not enough knowledge that explains the mechanism of RNA silencing functioning in *Heterobasidion* in response to infection by different partitivirus strains.

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